# Highly conserved modified nucleosides influence $Mg^{2+}$-dependent tRNA folding

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## ABSTRACT

**Transfer RNA structure involves complex folding interactions of the TΨC domain with the D domain. However, the role of the highly conserved nucleoside modifications in the TΨC domain, $rT_{54}$, $\Psi_{55}$ and $m^5C_{49}$, in tertiary folding is not understood. To determine whether these modified nucleosides have a role in tRNA folding, the association of variously modified yeast $tRNA^{Phe}$ T-half molecules (nucleosides 40–72) with the corresponding unmodified D-half molecule (nucleosides 1–30) was detected and quantified using a native polyacrylamide gel mobility shift assay. $Mg^{2+}$ was required for formation and maintenance of all complexes. The modified T-half folding interactions with the D-half resulted in $K_d$s ($rT_{54}$ = 6 ± 2, $m^5C_{49}$ = 11 ± 2, $\Psi_{55}$ = 14 ± 5, and $rT_{54},\Psi_{55}$ = 11 ± 3 μM) significantly lower than that of the unmodified T-half (40 ± 10 μM). However, the global folds of the unmodified and modified complexes were comparable to each other and to that of an unmodified yeast $tRNA^{Phe}$ and native yeast $tRNA^{Phe}$, as determined by lead cleavage patterns at $U_{17}$ and nucleoside substitutions disrupting the Levitt base pair. Thus, conserved modifications of tRNA's TΨC domain enhanced the affinity between the two half-molecules without altering the global conformation indicating an enhanced stability to the complex and/or an altered folding pathway.**

## INTRODUCTION

All species of cytoplasmic tRNA appear to have the same basic L-shaped tertiary structure. The anticodon stem and loop and the helical aminoacyl stem of the cloverleaf secondary structure remain independent of the global tertiary fold. However, the loop containing dihydrouridine (D-loop), and that containing ribothymidine and pseudouridine (TΨC-loop) form the junction between these two physically separate domains (Fig. 1). Though tRNA nucleotide sequences are not

well conserved across kingdoms, some of tRNA's post-transcriptional modifications are highly conserved throughout all cytoplasmic tRNA species (1). Modifications in tRNA's TΨC domain represent some of the most highly conserved RNA modifications. Pseudouridine at position 55, $\Psi_{55}$, occurs at a frequency of 90%. Ribothymidine, or 5-methyluridine ($rT_{54}$), occurs at position 54 in 60% of all sequenced tRNAs, and $m^5C$ at positions 48, 49 or both in 22%, 11% and 6%, respectively (1). Conservation of the nucleoside modifications in the TΨC domain suggests that they may play an important role in a function common to all tRNAs or in the folding of tRNA into its conserved tertiary structure for proper function.

tRNA's complex folding, the result of long-range, tertiary structure hydrogen bonding and metal ion coordination between the D-, TΨC- and variable-loops, occurs soon after transcription and before processing of the tRNA is complete (2,3). The folding interactions of the conserved L-shaped structure of cytoplasmic tRNAs are characterized by hydrogen bonding of $G_{19}$ in the D domain with $C_{56}$ in the TΨC domain and by a $G_{18}$ base pair to $\Psi_{55}$ (4). The tRNA molecule does not need to be in its native, fully processed, mature size for modification enzymes to produce $rT_{54}$ and $\Psi_{55}$, yet some structure of the RNA molecule is required (5–7). While neither $rT_{54}$ nor $\Psi_{55}$ is required for tRNA aminoacylation, elongation factor recognition or codon recognition on the ribosome *in vitro* (8), *Escherichia coli* lacking the enzyme for $\Psi_{55}$ synthesis are at a disadvantage in competing with wild type cells (9). In addition, *E.coli* lacking the gene for $rT_{54}$ synthesis are not viable (10). However, the $rT_{54}$ synthesis activity does not appear to be the cause, since cells with inactive enzymes are viable (11).

tRNA's tertiary folding interactions *in vitro* are observed with transcripts completely devoid of modifications (12,13). Yet, thermal denaturation of the fully modified, native tRNA occurs at a higher $T_m$ than the corresponding unmodified transcript (12,14,15). Incorporation of $rT_{54}$ increased the $T_m$ of a 17mer analog of the yeast $tRNA^{Phe}$ TΨC stem and loop domain with a corresponding decrease in $\Delta G$, whereas $\Psi_{55}$ had no measurable effect on the melting temperature or free energy (7). Though the base-stacking contributions of $\Psi$ compared with uridine were significant in a base-paired region of tRNA (16–19), hydrogen bonding through pseudouridine's
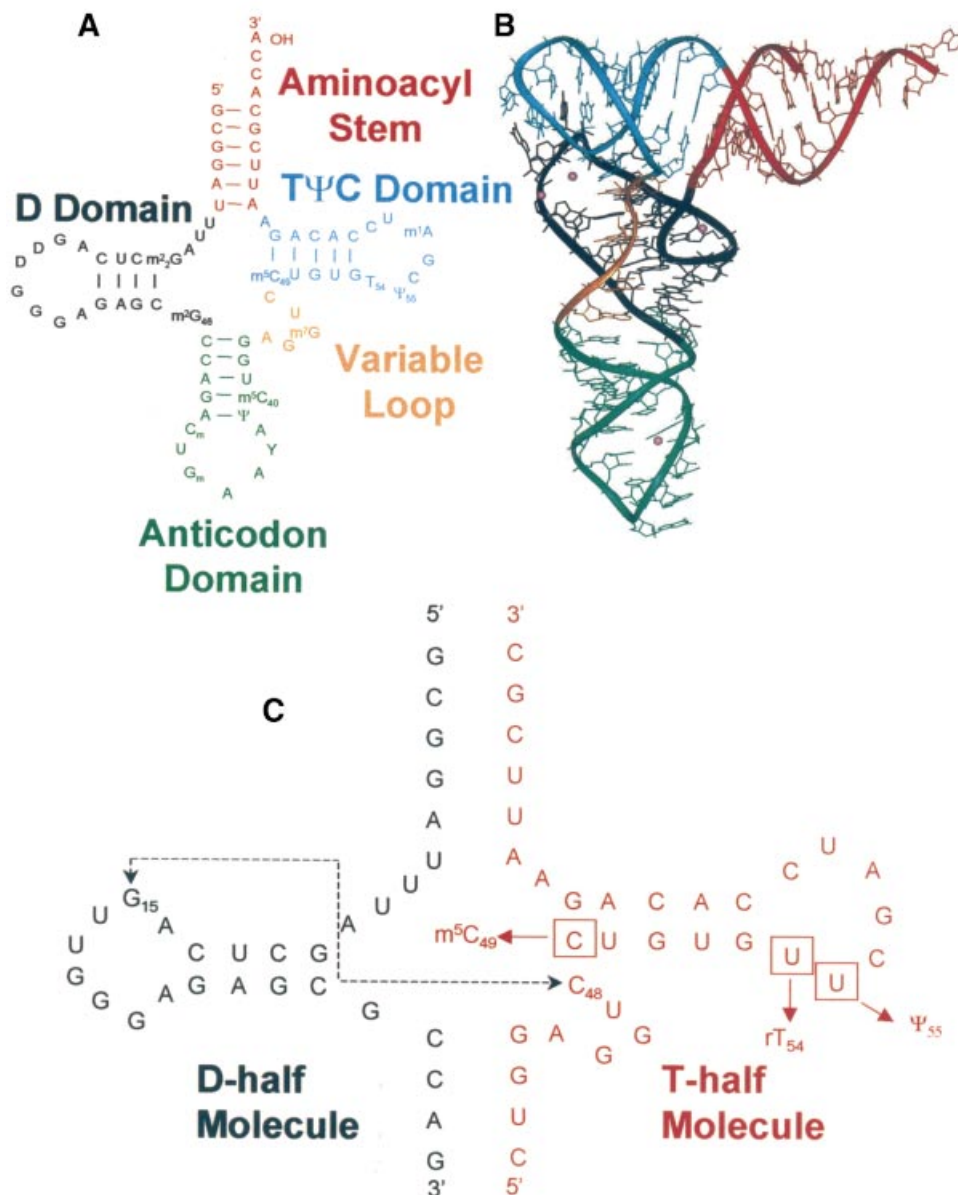
**Figure 1.** (**A**) Cloverleaf secondary structure of yeast tRNA[Phe] color-coded by domain. Red is the aminoacyl stem; blue is the T-stem–loop; orange is the variable loop; green is the anticodon stem–loop; and black is the D-stem–loop. (**B**) Crystal structure of yeast tRNA[Phe] (4) showing tertiary interactions between the T-loop and D-loop folding tRNA into its native L structure. Color coding corresponds to the secondary domains indicated in (A). The placement of $Mg^{2+}$ ions was determined by X-ray diffraction as reported previously and is indicated by pink spheres. (**C**) The unmodified sequence of chemically synthesized constructs that mimic the T domain and D domain in yeast tRNA[Phe]. Positions of incorporated modifications are boxed; $m^5C_{49}$, $m^5U_{54}$ ($rT_{54}$) and $\Psi_{55}$ were singly or doubly incorporated into the T-half molecule. The Levitt base pair in yeast tRNA[Phe] occurs between $G_{15}$ and $C_{48}$ and is indicated by a dashed line. Constructs altering the potential for a Levitt base pair contained a U at position 15 in the D-half molecule (D-half $G_{15}U$), or a U at position 48 in the T-half molecule (T-half $C_{48}U$).

N1 may be favored in tertiary interactions between loops. Therefore, $rT_{54}$ increases the thermal stability of tRNA at both the secondary (7,20) and tertiary level (21) and $\Psi_{55}$ provides base-stacking and/or hydrogen bonding contributions to the tertiary interaction with the D domain. However, the contributions of the individual modified nucleosides for the folding process, and not the resulting functional structure, are unclear. Perhaps the modifications affect the kinetics of tRNA's folding interactions and/or the number of folding pathways.

Empirical data on the contributions of individual modified nucleosides to the folding pathways for tRNA have been limited because experimental systems either utilize completely modified, native tRNA or completely unmodified transcripts (12,14,15). A structural study of the isolated stem and loop of the TΨC domain suggested a modification-dependent folding intermediate (20). The 17mer with the $rT_{54}$ modification adopted a structure similar to that of the same domain within the native tRNA's crystal structure, but distinctly different from the completely unmodified 17mer

RNA (22,23). This result, in addition to the folding interaction of $\Psi_{55}$ with the D domain, suggests that modifications in the T$\Psi$C domain may enhance the natural folding process of tRNAs.

The folding of RNA's highly negative phosphate backbone requires the presence of counterions, typically $Mg^{2+}$. Similarly, tRNA folding depends on the presence of counterions (12), but any contributions of $rT_{54}$ and $\Psi_{55}$ to this counterion dependence have yet to be determined. The counterion-dependent folding of native yeast tRNA$^{Phe}$ and the unmodified tRNA$^{Phe}$ transcript suggests that several folding pathways exist in which distinct, stable intermediate conformations can be observed (15,24). However, the site-specific coordination of $Mg^{2+}$ ions in tRNA has been challenged (25). Although $Mg^{2+}$ is coordinated between the D- and T$\Psi$C-loops within the crystal structure of yeast tRNA$^{Phe}$ (4,26,27), recent results with monovalent ions have suggested that electrostatic shielding of the phosphate backbone alone localizes $Mg^{2+}$ to certain regions of the tRNA without coordination to specific ligands (25). Modified nucleosides have been implicated in the specific coordination of $Mg^{2+}$ by native tRNAs (28,29) and in lowering the concentration of $Mg^{2+}$ required for tertiary structure interactions (28). In contrast to native tRNA, tertiary structure interactions of unmodified transcripts have an absolute requirement for either high millimolar concentrations of $Mg^{2+}$ (12–14) or molar concentrations of monovalent ion (15). However, the specific effect of $rT_{54}$ and $\Psi_{55}$ on $Mg^{2+}$ coordination in the tertiary structure has not been determined (13,30).

To better understand the role of modified nucleosides in the ion-dependent folding interactions of tRNA, we have developed an experimental bimolecular model system. The model system allows us to site-specifically incorporate the naturally occurring, highly conserved modified nucleosides and monitor a bimolecular interaction of two RNA fragments mimicking the T$\Psi$C domain and the D domain of yeast tRNA$^{Phe}$. The interaction of chemically synthesized half molecules, corresponding to nucleosides 1–30 (D-half) with nucleosides 40–72 (T-half) of yeast tRNA$^{Phe}$, was monitored in a quantifiable polyacrylamide gel mobility shift assay. Here, we report that formation of the complex by the two half molecules required $Mg^{2+}$ and introduction of the highly conserved modifications $rT_{54}$ and $\Psi_{55}$ and the commonly occurring $m^5C_{49}$ enhanced the affinity of the unmodified D-half molecule for the T-half molecule, but did not alter the global fold of the complex, which was similar to native yeast tRNA$^{Phe}$.

## MATERIALS AND METHODS

### RNA synthesis, purification and analysis

tRNA half molecules were chemically synthesized with the nucleotide sequence of yeast tRNA$^{Phe}$ (Fig. 1C) using 2′-bis(acetoxyethoxy)-methyl ether (ACE; Dharmacon, Inc., Boulder, CO) (31), 2′-*O*-triisopropylsilyl-oxy-methyl (TOM; Xeragon, AG, Zurich, Switzerland) (32) and standard phosphoramidite chemistries (NCSU Nucleic Acids Facility, Raleigh, NC) (33,34). The unmodified D-half molecule RNAs corresponded to the 30mer sequence of nucleotides 1–30 in

yeast tRNA$^{Phe}$ and the variously modified T-half RNAs corresponded to the 33mer sequence of nucleotides 40–72 in yeast tRNA$^{Phe}$ (Fig. 1). The 1–72 nt unmodified sequence of yeast tRNA$^{Phe}$ was chemically synthesized (Xeragon, AG) to mimic the completely unmodified yeast tRNA$^{Phe}$ transcript. RNAs were 3′-end labeled with [5′-$^{32}$P]Cp using RNA ligase (35) and then diluted with known quantities of unlabeled half molecule. Radiolabeled [5′-$^{32}$P]Cp was produced from [$\gamma$-$^{32}$P] ATP (Amersham Biosciences, Piscataway, NJ) and CMP using polynucleotide kinase (35).

### Gel mobility-shift assay

Gel mobility-shift assays were conducted with 15% native polyacrylamide gels composed of a Tris–borate buffer (TB; 89 mM Tris–HCl, 89 mM boric acid, pH 8.1). $Mg^{2+}$, present only in the gel-forming reaction and in TB running buffer, was at a concentration of 3 mM, unless noted. The electrophoresis apparatus was surrounded by ice to maintain a temperature <25°C. All 3′-end-labeled T-half molecules were purified by a 7 M urea denaturing polyacrylamide gel electrophoresis (PAGE) (15%) prior to reaction with the D-half molecule in the native mobility-shift assay. Purified T-half molecules, eluted from gels using denaturing elution buffer (0.5 M ammonium acetate, 1% SDS, 0.1 mM ethylenediamine-tetraacetic acid), were reacted with the unmodified D-half in 15 µl of native loading buffer (30% glycerol in 89 mM Tris–HCl, 89 mM boric acid, pH 8.1). A fixed concentration of the T-half molecule was titrated with increasing amounts of the D-half molecule in individual reaction mixtures as described in the figure legends. The reaction mixtures were heated at 65°C for 10 min (in the absence of $Mg^{2+}$), then allowed to cool to room temperature prior to loading on the gel. After electrophoresis, the gel was dried and visualized by phosphorimaging (36). T-half molecules free and within the T-half/D-half complex were quantified by analyzing the volume of the phosphorimage using ImageQuant software (Molecular Dynamics, Amersham Biosciences). Only the two prominent bands, free and bound, were analyzed. The material between the two bands was not included in the analysis of free or bound material (see Fig. 5). A lane containing no RNA was used for a background subtraction from all lanes with bound and free RNA. The percent complex formation was determined from the volumes and then normalized to 100% for relative $K_d$ determination. The data were fitted to a one-site binding model using Prism software and errors were based on the deviations from the least squares fit (GraphPad Software, San Diego, CA).

### Ribonuclease T1 and lead cleavage reactions

The 3′-end-labeled RNAs and RNA complexes (D- and T-half molecule complexes) were purified by PAGE (15%). The RNAs were identified on the gel by ethidium bromide staining. Individual RNAs were eluted from the gel in denaturing elution buffer (0.5 M ammonium acetate, 1% SDS, 0.1 mM EDTA), whereas complexes of the D- and T-half molecules were eluted with native elution buffer (20 mM HEPES, 250 mM KCl and 5 mM MgCl$_2$). The eluted RNAs were ethanol precipitated, dried and resuspended to a concentration of 20 µM RNA in water. RNAs that were not in complexes were denatured and renatured by heating to 70°C for 5 min and slowly cooled to room temperature prior to use.
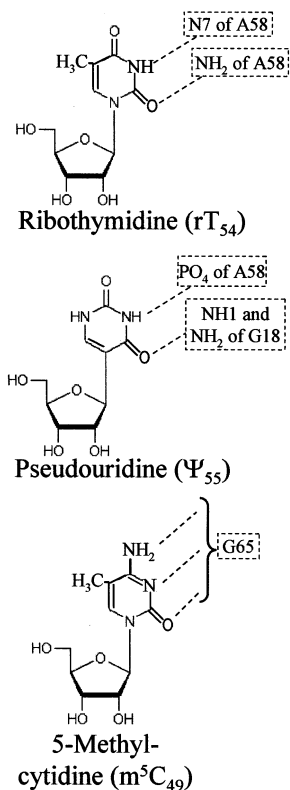
Ribothymidine (rT$_{54}$)

Pseudouridine ($\Psi_{55}$)

5-Methyl-cytidine (m$^5$C$_{49}$)

**Figure 2.** The chemical structure and hydrogen bonding of the conserved modifications, rT$_{54}$, $\Psi_{55}$ and m$^5$C$_{49}$. Modified T-half molecule constructs were chemically synthesized with the modified nucleosides rT$_{54}$, $\Psi_{55}$ and m$^5$C$_{49}$ individually, or with both rT$_{54}$ and $\Psi_{55}$ (Fig. 2). The hydrogen bonding of each of these modified nucleosides as found in the X-ray crystallographic tertiary structure yeast tRNA$^{Phe}$ is shown in the figure. Ribothymidine or 5-methyluridine (rT$_{54}$) forms a tertiary structure base pair with m$^1$A$_{58}$. However, our T-half molecules are constructed with the unmodified A$_{58}$. Methyl-5-cytidine forms a canonical Watson–Crick base pair with G$_{65}$.

Lead cleavage reactions (10 µl) contained 4 µM RNA (40 mM NaCl, 10 mM Tris–HCl, pH 7.2, 10 mM MgCl$_2$) and 0.5 mM Pb(II) acetate. Reaction mixtures were stored on ice, initiated by the addition of Pb(II) acetate and incubated at 20°C for 15 min. The lead cleavage sites were mapped by generating an alkaline hydrolysis ladder. Alkaline hydrolysis was conducted by heating reaction mixtures (10 µl) containing 2 µM RNA and formamide (1:5 by volume in 50 mM NaHCO$_3$/Na$_2$CO$_3$ pH 9.1) at 100°C for 10 min. In addition, the three Gs (G$_{18}$, G$_{19}$ and G$_{20}$) in the D-loop that are 3′ to the expected lead cleavage site at U$_{17}$, were mapped with RNase T1 digestion (Promega, Madison, WI). RNase T1 partial digestions were performed in reaction mixtures (10 µl) containing 2 µM RNA (50 mM sodium citrate, 4.5 M urea) with 5 U RNase T1 at 55°C for 20 min. Cleavage reactions were stopped by addition of 10 µl formamide loading dye (100% deionized formamide, 10 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF). Radiolabeled products of the cleavage reactions were analyzed by 7 M urea–PAGE (15%) and visualized by phosphorimaging.
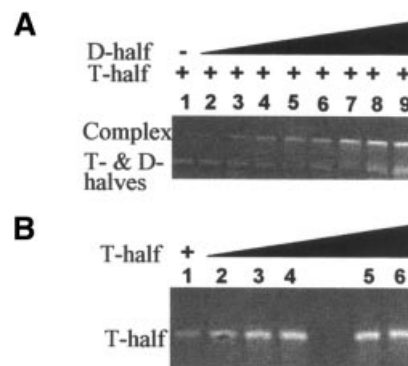


**Figure 3.** Complex formation between the unmodified D- and T-half molecules was detected in a native PAGE (15%, 3.0 mM Mg$^{2+}$) with ethidium bromide staining. Free D- and T-half molecules migrated similarly, and stained less well than the slower migrating complex. (**A**) Lanes 1–9 contained 5.6 µM unmodified T-half molecule. Lanes 2–9 contained increasing amounts of unmodified D-half molecule with the following µM concentrations: 0.56, 1.68, 2.81, 4.21, 5.61, 16.8, 56.1 and 112. (**B**) Increasing amounts of unmodified T-half molecule alone in a native PAGE (15%, 3.0 mM Mg$^{2+}$) demonstrated that the half molecule does not aggregate. Lanes 1–6 contained unmodified T-half with the following µM concentrations: 1.6, 3.0, 4.2, 5.6, 7.0 and 8.4.

## RESULTS

### Design of a bimolecular system to mimic tRNA folding

A bimolecular experimental model was designed to determine the contributions of modified nucleosides to the folding interaction of tRNA's TΨC domain with the D domain. The experimental system consisted of a 33 nt T-half molecule and a 30 nt D-half molecule (Fig. 1C). The T-half molecule was synthesized unmodified or with the modified nucleosides rT$_{54}$, $\Psi_{55}$, m$^5$C$_{49}$ or with both rT$_{54}$ and $\Psi_{55}$ (Fig. 2). While a previous study had used a similar bimolecular model to determine the minimal fragments of yeast tRNA$^{Phe}$ required for leadzyme activity (37), we extended the lengths of our half molecules to include the aminoacyl stem and anticodon stem base-paired regions (Fig. 1C). Though a 26 nt D-half formed a complex with a 29 nt T-half molecule, an extension to slightly larger RNAs was necessary to achieve complete T- and D-half molecule complex formation as a gel mobility shift under the native PAGE conditions used in this study (Fig. 3). Mobilities of RNAs in native gels are apparently sensitive to molecular conformation and Mg$^{2+}$ concentrations (38). In the presence of 3 mM Mg$^{2+}$ and increasing concentrations of the D-half molecule, the intensity of the faster moving band of individual half molecules decreased with a concomitant increase in the intensity of the slower moving complex (Fig. 3A, lanes 2–5). An increase in the intensity of the faster moving band was observed with addition of excess D-half molecule (Fig. 3A, lanes 6–9). Complex formation required the presence of both half molecules. No gel shift was evident with either half molecule alone, even at 10-fold higher concentrations indicating that the individual half molecules did not aggregate (Fig. 3B).

Complex formation also required Mg$^{2+}$ in the gel and running buffer. Mg$^{2+}$ in the sample alone was not sufficient to demonstrate complex formation by a gel shift (Fig. 4A). At least 0.75 mM Mg$^{2+}$ was required for a complex to form
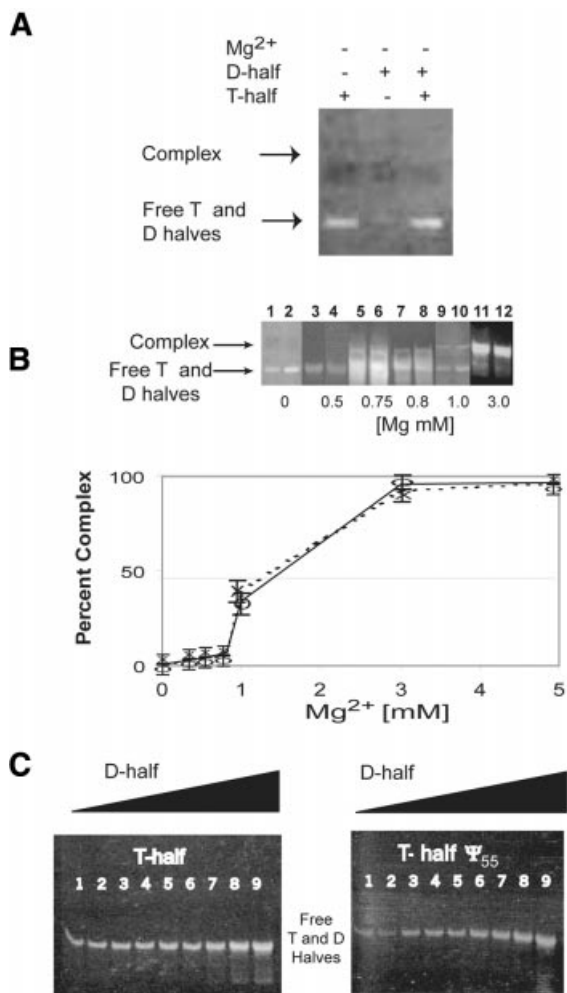
**Figure 4.** (**A**) The absence of $Mg^{2+}$ in both the running buffer and gel precluded complex formation. A native polyacrylamide gel (15%) was polymerized in the absense of $Mg^{2+}$. The running buffer (TB) also lacked $Mg^{2+}$. RNA was detected with ethidium bromide staining. Samples for lanes 1 and 2 of the gel contained 84.2 µM unmodified T-half and D-half, respectively, but the D-half did not stain well with ethidium bromide, due to lack of secondary structure (John Stuart and Paul F. Agris, personal communication). Lane 3 contained 84.2 µM unmodified T-half and 84.2 µM D-half. (**B**) A $Mg^{2+}$ concentration >0.75 mM is required for complex formation. Complex formation was analyzed under different $Mg^{2+}$ concentrations from 0 to 5 mM. Samples of 84.2 µM unmodified (lanes 1, 3, 5, 7, 9 and 11), or Ψ-containing T-half (lanes 2, 4, 6, 8, 10 and 12) and 84.2 µM D-half molecules were subjected to native PAGE in which the gel, sample and running buffers contained the same concentration of $Mg^{2+}$. The composite of gels depicts examples of the results used to produce the graph below (unmodified T-half, open circles, solid line; Ψ-containing T-half, cross, dotted line). (**C**) The left-hand native gel demonstrates a lack of complex formation for the unmodified T-half molecule with increasing concentrations of the D-half molecule in the presence of only 0.5 mM $Mg^{2+}$. Lane 1 contained 84.2 µM unmodified T-half molecule. Lanes 2–9 contained 84.2 µM unmodified T-half with the following µM concentrations of D-half: 8.42, 25.3, 42.1, 63.2, 84.2, 253, 842 and 1684 µM. The right-hand native gel demonstrates a lack of complex formation for the $Ψ_{55}$-containing T-half molecule in the presence of 0.5 mM $Mg^{2+}$. Lane 1 contains 84.2 µM $Ψ_{55}$ T-half alone. Lanes 2–9 contain 84.2 µM $Ψ_{55}$ T-half with the following µM concentrations of D-half: 8.42, 25.3, 42.1, 63.2, 84.2, 253, 842 and 1684 µM.

whether the T-half was unmodified or had $Ψ_{55}$ (Fig. 4B). A 3 mM concentration of $Mg^{2+}$ was determined to be at saturation for complex formation (Fig. 4B). Since $Ψ_{55}$, the
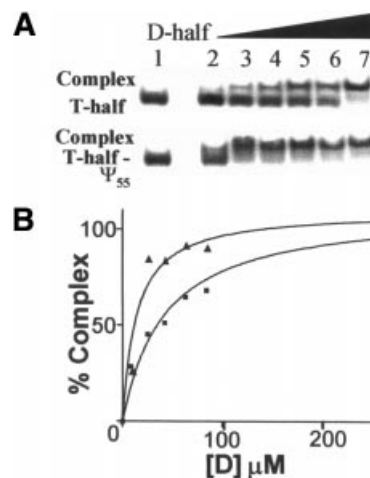


**Figure 5.** (**A**) Mobility-shift assays of $^{32}P$-end-labeled, unmodified and modified, T-half molecules with increasing amounts of unmodified D-half molecule. In the native PAGE (15%, 3.0 mM $Mg^{2+}$) at the top of the figure, complex formation is detected when the unmodified, $^{32}P$-labeled T-half molecule interacts with the unmodified D-half molecule. Below, a complex between the $Ψ_{55}$-containing T-half molecule (T-half-$Ψ_{55}$) with the unmodified D-half molecule is detected. All lanes contained 84.2 µM T-half molecule. Lanes 2–7 of each gel contained increasing amounts of unmodified D-half molecule as follows: 8.42, 25.3, 42.1, 63.2, 84.2 and 253 µM, respectively. (**B**) Percent complex formed versus concentration of the D-half molecule for the unmodified D-half molecule with unmodified T-half molecule (closed square) or with the $Ψ_{55}$-containing T-half molecule (closed triangle). Data were normalized to 100% complex formation and curves were fitted to the data with a non-linear regression assuming one-site binding. The percent complex was determined by quantifying the phosphorimages from the PAGE in (A) using ImageQuant software (Molecular Dynamics) and errors were determined as deviations from the least squares fit.

most highly conserved of the three T-half modifications, did not lower the requirement for $Mg^{2+}$ (Fig. 4B), experiments were routinely conducted with 3 mM $Mg^{2+}$ to ensure that the effects of modifications, and not of $Mg^{2+}$, were being measured.

A gel mobility shift, indicating complex formation of the T- and D-half molecules, was observable with ethidium bromide staining (3 mM $Mg^{2+}$, pH 8.1, 25°C). The individual T- and D-half molecules, though of different lengths and sequences, exhibited very similar migrations under native PAGE conditions. However, labeling either half molecule with $^{32}P$ allowed us to observe that half molecule as free or as participating in the complex (Fig. 5). The complex was detected independently of which half molecule was labeled, but the T-half molecule was routinely labeled for all quantified experiments. The integrity of the complex could be maintained during extraction from the gel in the presence of $Mg^{2+}$. Upon removal of $Mg^{2+}$, the complex dissociated into the individual D- and T-half molecules (data not shown).

## TΨC domain modifications enhanced affinity of the D-half molecule for the T-half molecule

The binding affinities of the unmodified and variously modified T-half molecules for the completely unmodified D-half molecule were compared. Binding affinities for the interacting half molecules were determined by titrating the 3′-$^{32}P$-labeled T-half molecules with increasing concentrations

**Table 1.** Binding affinities and free energy for complex formation between the unmodified D-half and variously modified T-half molecules

| T-half molecule[a] | $K_d$ (μM)[b] | $\Delta G_{37}$[c] |
|---|---|---|
| Unmodified | $40 \pm 10$ | $-6.2 \pm 1.6$ |
| $RT_{54}$ | $6 \pm 2$ | $-7.4 \pm 2.0$ |
| $m^5C_{49}$ | $11 \pm 2$ | $-7.0 \pm 1.3$ |
| $\Psi_{55}$ | $14 \pm 5$ | $-6.9 \pm 2.5$ |
| $RT_{54};\Psi_{55}$ | $11 \pm 3$ | $-7.0 \pm 1.9$ |

[a]T-half molecule constructs containing the site-specific incorporations of the indicated modified nucleosides.
[b]Binding constants were determined by quantifying mobility shift assays (Materials and Methods), plotting % complex formation versus concentration of D-half molecule, and fitting data with non-linear regression.
[c]$\Delta G_{37}$ (kcal/mol at 37°C) was calculated from the association constants ($\Delta G = -RT \ln K$).

of the unlabeled D-half molecule (Fig. 5). Free and complexed T-half molecules were separated by native PAGE and quantified by phosphorimaging, as described in Materials and Methods. Binding affinity, calculated as dissociation constants ($K_d$), varied with the modification present in the T-half molecule (Table 1). All $K_d$ values were determined under saturating conditions although, for clarity, binding isotherms are not shown to saturation (Fig. 5). While all of the naturally occurring TΨC domain modifications that we investigated, $m^5C_{49}$, $T_{54}$ and $\Psi_{55}$, enhanced the affinity of the half molecules, the $rT_{54}$-containing T-half molecule exhibited the highest affinity for the unmodified D-half molecule (Table 1). The T-half molecule with rT site-specifically incorporated at its naturally occurring position, 54, exhibited an affinity for the D-half molecule, 7-fold higher ($K_d = 6 \pm 2$ μM) than that of the unmodified T-half molecule ($K_d = 40 \pm 10$ μM). Pseudouridine at position 55 increased the binding affinity of the T-half molecule ~3-fold ($K_d = 14 \pm 5$ μM), compared with the unmodified T-half molecule. The $m^5C_{49}$ modification increased the affinity of the T-half almost 4-fold ($K_d = 11 \pm 2$ μM) compared with that of the unmodified T-half molecule. A doubly modified T-half molecule containing both $rT_{54}$ and $\Psi_{55}$ exhibited an affinity ($K_d = 11 \pm 3$ μM) comparable with that of the $m^5C_{49}$-containing T-half molecule and ~4-fold higher than that of the completely unmodified T-half molecule. The affinity of the $rT_{54}$ and $\Psi_{55}$ modified T-half molecule for the D-half was intermediate to that of the $rT_{54}$ or $\Psi_{55}$ modification alone.

## Modifications do not lower the $Mg^{2+}$ requirement for complex formation

Binding affinities were determined by mobility-shift assays conducted under excess $Mg^{2+}$ concentrations to minimize any contributions the modified nucleosides may have had in coordinating $Mg^{2+}$. In order to determine if the most highly conserved modification in tRNA (1), $\Psi_{55}$, altered the $Mg^{2+}$ requirement, native PAGE mobility-shift assays were conducted at various concentrations of $Mg^{2+}$ (0–5 mM). The unmodified T-half and $\Psi_{55}$-containing T-half molecules were titrated with the unmodified D-half molecule. The minimum $Mg^{2+}$ concentration required for the observable interaction between the two unmodified half molecules was ~0.75 mM (Fig. 4B). In the presence of 0.5 mM $Mg^{2+}$, even a 20-fold concentration of D-half to T-half molecule failed to generate
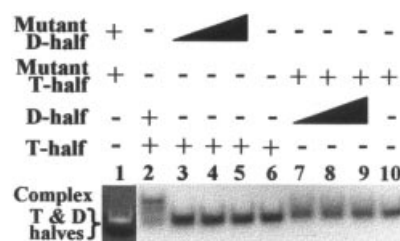


**Figure 6.** Absence of complex formation by T- and D-half molecules without the potential for Levitt base-pair. Lane 1 contained 84.2 μM of unlabeled T-half $C_{48}U$ and 84.2 μM D-half $G_{15}U$. The single RNA band, visualized with ethidium bromide staining, is that of the free T-half $C_{48}U$ and D-half $G_{15}U$ molecules that migrate similarly in the gel. A mobility shift indicating complex formation was not detectable. Lane 2 contained the positive control of unmodified T-half molecule (84.2 μM) with unmodified D-half (84.2 μM). The major band was the mobility-shifted, $^{32}$P-end-labeled T-half molecule in complex with D-half molecule. The RNA was visualized by phosphorimaging. Some free T-half molecule is also observed. Lanes 3–5 contained $^{32}$P-end-labeled unmodified T-half molecule (84.2 μM) with an increasing concentration of the D-half $G_{15}U$, 8.42, 84.2 and 253 μM, respectively. A Levitt base pair interaction by the half molecules in lanes 3–5 would require a $U_{15}C_{48}$ base pair to occur. Lane 6 contained $^{32}$P-end-labeled, unmodified T-half molecule (84.2 μM) only. Lanes 7–9 contained the T-half $C_{48}U$ molecule (84.2 μM) with increasing amounts of unmodified D-half molecule, 8.42, 84.2 and 253 μM, respectively. A Levitt base pair interaction by these half molecules would require formation of a $G_{15}U_{48}$ base pair. Lane 10 contained 84.2 μM $^{32}$P-end-labeled $C_{48}U$ T-half molecule, alone. Mobility of the free T-half molecule varied slightly across the gel.

an observable complex formation (Fig. 4C). Incorporation of $\Psi_{55}$ into the T-half molecule did not lower the $Mg^{2+}$ concentration required to observe the mobility-shift (Fig. 4B and C). Additions of very small amounts of $Mg^{2+}$ readily alter the conformation of tRNA$^{Phe}$, as revealed by hydroxy radical cleavage patterns (39). Therefore, while the conserved modifications enhanced the affinity of the T-half molecule for the D-half molecule, they did not lower the $Mg^{2+}$ requirement for the interaction of the two half molecules under the conditions of the assay.

## Comparison of global folds of the D and TΨC complex and native yeast tRNA$^{Phe}$

Yeast tRNA$^{Phe}$ has a $G_{15}$:$C_{48}$ tertiary structure base pair in common with some 65% of all tRNAs (1,40). This base pair, known as the Levitt pair, occurs between $G_{15}$, in the D-loop, and $C_{48}$, in the variable loop, in yeast tRNA$^{Phe}$ (Fig. 1C). To ensure that complex formation observed under native PAGE conditions was a result of known tertiary structure interactions between D- and T-half molecules, and not simply a result of canonical base-pairing between the aminoacyl and anticodon stems, RNAs were synthesized to eliminate the possibility of the $G_{15}$:$C_{48}$ Levitt tertiary structure base pair. D- and T-half molecules with U substitutions in both positions 15 (D-half $G_{15}U$) and 48 (T-half $C_{48}U$) would not be expected to form a complex, and none was observed (Fig. 6). No complex was observed when the completely unmodified, native sequence T-half molecule was combined with the D-half $G_{15}U$, since a $U_{15}$:$C_{48}$ base pair would have to form (Fig. 6). However, when the unmodified, but native sequence D-half molecule was incubated with the T-half $C_{48}U$ molecule, a small amount of RNA with the mobility of the native sequence D-half/T-half complex and a faster moving, diffuse band of RNA in the gel
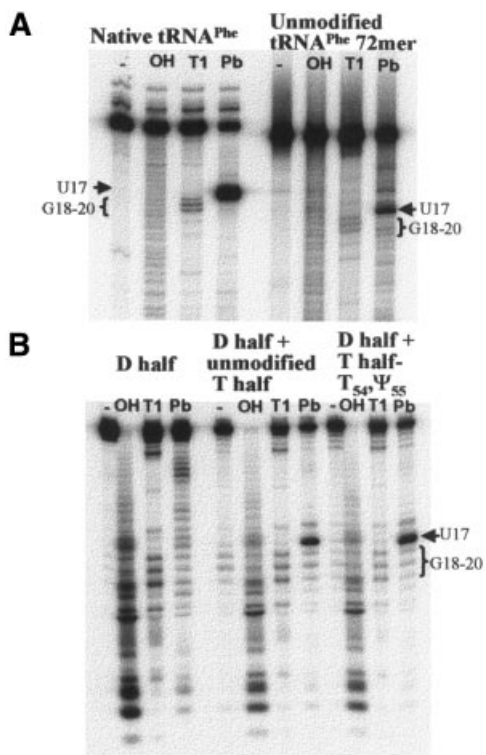
**Figure 7.** Lead, Pb(II), cleavage of native tRNA$^{Phe}$ and the D-half molecule in the presence of the unmodified and the rT$_{54}$ and Ψ$_{55}$ modified T-half molecules produced a distinct cleavage pattern not observed in the D-half molecule alone. (**A**) Native tRNA$^{Phe}$ and unmodified tRNA$^{Phe}$ 72mer, and (**B**) free D-half molecule, the complex from unmodified D- and T-half molecules and that from the unmodified D-half with the rT$_{54}$ and Ψ$_{55}$ modified T-half molecule were $^{32}$P-3′-end-labeled and subjected to: (−) no treatment, (OH) alkaline hydrolysis, (T1) T1 nuclease and (Pb) 0.5 mM Pb$^{2+}$. Alkaline hydrolysis and T1 nuclease treatments were used to confirm cleavage sites. All reactions are described in Materials and Methods. Reaction products were separated by 10% PAGE–7 M urea and visualized by phosphorimaging. The positions of the nucleotides sensitive to T1 cleavage (G18–20) and Pb$^{2+}$ cleavage (between U16 and U17) are indicated in the figure. The heavy bands at the top of the lanes in (A) and (B) are the full-length native tRNA$^{Phe}$ and the unmodified, 72mer analog.

were observed. The diffuse band of RNA in the gel could have resulted from a G$_{15}$:U$_{48}$ tertiary interaction plus tertiary structure mis-pairings that yielded complexes with many conformations (Fig. 6). Observation of a mobility-shift that required the two half-molecule constructs, the Levitt base pair and Mg$^{2+}$ was indicative of the complex having a global fold similar to that of native yeast tRNA$^{Phe}$. To confirm that the overall structure of the complex composed of the T- and D-half molecules was indeed similar to that of native tRNA$^{Phe}$, we took advantage of the tRNA's ability to self-cleave with Pb$^{2+}$ only when in the native fold (41). A T-half/D-half complex with a fold similar to that of native tRNA$^{Phe}$ should exhibit the well-characterized self-cleavage reaction catalyzed by lead (37). When correctly folded native tRNA$^{Phe}$ acts as a leadzyme, a specific and strong, single site cleavage of the D-loop between D$_{17}$ (or U$_{17}$) and G$_{18}$ results in identifiable fragments (Fig. 7A). RNase T1 digestions, under denaturing conditions, and alkaline hydrolysis ladders confirmed the location of the lead cleavage (Fig. 7A). The location of the lead cleavage produced by an unmodified, chemically

synthesized and slightly truncated tRNA$^{Phe}$ (nucleotides 1–72) was identical to that of the native tRNA (Fig. 7A), as reported previously for an unmodified transcript (37). A specific self-cleavage by lead between U$_{17}$ and G$_{18}$ was achieved with the complex composed of the unmodified half molecules indicating that the global fold of the complex was similar to that of native tRNA$^{Phe}$ (Fig. 7B). Lead cleavage of the D-half molecule alone revealed random cleavages quite similar to that of an alkaline hydrolysis ladder, and dramatically different to the site-specific cleavage when complexed with the T-half molecule (Fig. 7B). Since the TΨC domain modifications enhanced the interaction of the T-half molecule for the unmodified D-half molecule, the modifications may also alter the structure of the resulting complex. Therefore, we performed the lead cleavage analysis to determine if modifications influenced the overall tertiary conformation of the complex. The strong, site-specific lead cleavage of a complex composed of the unmodified D-half and a rT$_{54}$,Ψ$_{55}$-containing T-half molecule was similar to that of the unmodified D-half and T-half complex (Fig. 7B). Thus, the lead cleavage patterns of the complexes, native yeast tRNA$^{Phe}$, and the unmodified analog of yeast tRNA$^{Phe}$, were all similar.

## DISCUSSION

The contributions of some highly conserved modifications to the folding of tRNA have not been well documented, although the Mg$^{2+}$-dependent folding of both unmodified and fully modified tRNAs has been studied (12,13,24). With tRNA containing the largest and most variant number of modified nucleosides, there may be a role for modifications in the folding process of tRNA by either stabilizing intermediates or altering local conformations for the overall folding of the tRNA into its native tertiary structure. In particular, site-specifically conserved modifications, such as rT$_{54}$ and Ψ$_{55}$, may be important for tRNA to efficiently and effectively achieve functional conformation under physiological conditions. To assess the roles of highly conserved, modified nucleosides and Mg$^{2+}$ in the folding interactions of tRNA's domains, we developed an experimental bimolecular model. Tertiary interactions of the TΨC domain with the D domain in the unimolecular yeast tRNA$^{Phe}$ were mimicked by the interactions of half-molecules. Incorporation of the highly conserved modifications rT$_{54}$ and Ψ$_{55}$, or the commonly occurring m$^5$C$_{49}$, enhanced the affinity of the T-half molecule with the unmodified D-half. However, Ψ$_{55}$ did not lower the Mg$^{2+}$ requirement for this interaction. Modifications in the T-half molecule did not affect the global conformation of the resulting complexes. Taken together, the enhanced affinity of the modified T-half molecule for the unmodified D-half and the early events of rT$_{54}$ and Ψ$_{55}$ syntheses in pre-tRNA (3) indicate that these conserved modifications contribute to the native TΨC and D domain folding interactions.

The TΨC domain folding interaction with the D domain required both the Levitt tertiary structure base pair present in the native yeast tRNA$^{Phe}$ and the presence of Mg$^{2+}$. No complex was observed in our gel mobility shift analysis when base substitutions completely negated the Levitt pair. Disruption of the Levitt base pair, as well as all D- to T-loop tertiary structure interactions, in an effort to define a functional, minimal sequence tRNA$^{Leu}$ analog, completely

negated aminoacylation by the *E.coli* leucyl-tRNA synthetase (42). Thus, formation of the Levitt pair may define one of the lowest energy conformations in the RNA's folding landscape (43) on the path to the native global fold. In tRNA's three-dimensional structure (4,26), the highly charged phosphate backbone folds sharply at the TΨC and D domain interface. The requirement for $Mg^{2+}$ could be explained by neutralizing electrostatic repulsion (44,45), in particular the large local negative charge at this interdomain interface (24,25). Though high concentrations of $Mg^{2+}$ were required, the unmodified yeast tRNA$^{Phe}$ transcript exhibited, in NMR spectra, the tertiary and secondary structure hydrogen bonding similar to that of the native tRNA and expected of a folding interaction between the TΨC and D domains (13). Results of Levitt base pair substitutions and site-specific lead cleavages reported here are consistent with the NMR investigation. Cleavage of native tRNA$^{Phe}$, an unmodified analog of tRNA$^{Phe}$, the complex formed from the unmodified D- and T-half molecules and the complex formed from the unmodified D-half molecule with variously modified T-half molecules all produced similar fragmentation patterns (Fig. 7). A $Mg^{2+}$ requirement for the bimolecular folding interaction in our model system is also consistent with electrostatic interactions dominating the free energy of RNA folding (46). The result is that stable tertiary structures of RNAs are observed only in the presence of divalent metal ions or with very high concentrations of monovalent ions (46).

Our bimolecular model system allows investigation of the contributions of individual modifications to interdomain contacts and folding interactions within tRNA. As with other RNAs, the folding process of tRNA involves a series of early and late folding events (46,47), secondary and tertiary interactions (48), as it is being transcribed in the cell and is then processed as pre-tRNA to the modified and sized, functioning molecule. Initiation of the early, secondary interactions in the tRNA folding process is likely to occur in a time scale (10–100 μs) approximately 1000 times more rapid than the late-occurring, tertiary interactions (49,50). Support that our model system is reporting the late occurring, specific tertiary interactions, is that the $\Delta G$ values observed in our model system (Table 1) are of a similar magnitude for interdomain contacts in other RNAs (1.7 ± 2 to 7.6 ± 1.2 kcal/mol) (51). In addition, the specific lead cleavage pattern and the presence of the Levitt base-pair in the completely unmodified D- and T-half molecule suggests that the contributions of modifications are to stabilize, rather than to form, the tertiary contacts.

Modifications in the TΨC domain possibly enhance the correct tertiary structure contacts for tRNA folding interactions. Thus, unproductive pathways and stable, but misfolded, conformations that have been observed in unmodified RNAs (52) are thwarted. The $m^1A_9$-dependent folding of a mitochondrial tRNA that has a small D domain loop (53) may be an example of a modified nucleoside required for a late folding event to the native structure. However, one of the most common modifications, $rT_{54}$, probably contributes to an early secondary structure alteration of the TΨC domain conformation such that the native folding interaction with the D domain would be affected. $rT_{54}$'s ability to stabilize and affect the local conformation of the T stem and loop (7,20), and the tRNA$^{fMet}$ species from *E.coli* and *Thermus thermophilus* (21),

indicates that enhanced secondary structure interactions could be responsible for our observation of the higher affinity of the modified T-half molecule than the unmodified for the D-half. The fact that catalytically inactive yeast $m^5U_{54}$-tRNA methyltransferase is required for stabilizing at least one of the cell's tRNAs, tRNA$^{Ser}_{CGA}$ (11), indicates that the modification enzyme may play a role in tRNA folding prior to catalyzing formation of $rT_{54}$. After methylation of $U_{54}$, the resulting folded structure would be retained through the stabilizing contributions of the modification.

In contrast to $rT_{54}$, $\Psi_{55}$ may directly favor tertiary interactions between the TΨC domain with the D domain without contributing secondary interactions to the TΨC domain alone. The presence of $\Psi_{55}$ in the otherwise unmodified 17mer TΨC domain did not further stabilize the RNA (7), but the presence of $\Psi$ in base-paired regions provides stability to RNA (16–19,54). $\Psi_{55}$ may not contribute to local stability of the TΨC domain, as does $T_{54}$. However, $\Psi$'s additional hydrogen bonding and/or base stacking capabilities (16–19) would understandably favor tertiary interactions between the TΨC domain and the D domain. Compared with the unmodified T-half molecule, the T-half molecule with $\Psi_{55}$ had a higher affinity for the D-half molecule. Introduction of $\Psi_{55}$ into the T-half molecule resulted in an additional –0.7 kcal/mol of free energy for the association of the two half molecules. Similarly, the introduction of the conserved $\Psi$ in snRNA stabilized that RNA's interaction with an intron branch point by –0.7 kcal/mol (55). These results, together with our finding that the unmodified D-half molecule's affinity for the doubly modified T-half $rT_{54};\Psi_{55}$ ($K_d = 11 \pm 3$ μM) was less than that for the singly modified T-half ($rT_{54}$, $K_d = 6 \pm 2$ μM), yet greater than that of the $\Psi_{55}$-modified T-half ($K_d = 14 \pm 5$ μM) and the unmodified T-half ($K_d = 40 \pm 10$ μM), suggest that $rT_{54}$ and $\Psi_{55}$ could play different roles in stabilizing the tertiary interactions in tRNA. The contributions of these individual modifications were not additive in our experimental model. When the other, adjacent, modification was present, each could influence the contributions of the other to the RNAs' folding interactions.

Recent theoretical and empirical approaches to folding describe the folding interactions of RNA molecules with $Mg^{2+}$ as one of loose and diffusely bound hydrated $Mg^{2+}$ ions participating in a rapid, non-specific collapse of the RNA into an intermediate that then finds, within this restricted conformational space, its stable native conformation (56,57). The enhanced folding interaction observed when conserved modifications are present in the TΨC domain may be indicative of modifications reducing conformational space and thus affecting the folding pathway and/or kinetics of reaching the native structure. We have observed by NMR that additions of a modification to an already modified yeast tRNA$^{Phe}$ anticodon stem and loop domain restricted its conformational space accessible in the direction of the native structure (J.W.Stuart and P.F.Agris, unpublished results). Although we have as yet to identify a stable folding intermediate by native PAGE under conditions of low $Mg^{2+}$ concentration, the application of urea titrations (14,15) and circular dichroism studies to our bimolecular approach may yield information about modified nucleoside contributions to the formation of specific intermediates in the tertiary folding of tRNA. Because tRNA processing enzymes and the

translation machinery of the cell are sensitive to the tertiary fold of tRNA, effects of modification on the pathway and rate of tRNA folding and unfolding could determine an organism's ability to survive (58,59).

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